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PUSHING THE FRONTIERS OF RADIOBIOLOGY: A SPECIAL FEATURE IN MEMORY OF SIR OLIVER SCOTT AND PROFESSOR JACK FOWLER: REVIEW ARTICLE

The tumour microenvironment links complement system dysregulation and hypoxic signalling

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ABSTRACT

The complement system is an innate immune pathway typically thought of as part of the first line of defence against "non-self" species. In the context of cancer, complement has been described to have an active role in facilitating cancer-associated processes such as increased proliferation, angiogenesis and migration. Several cellular members of the tumour microenvironment express and/or produce complement proteins locally, including tumour cells. Dysregulation of the complement system has been reported in numerous tumours and increased expression of complement activation fragments in cancer patient specimens correlates with poor patient prognosis. Importantly, genetic or pharmacological targeting of complement has been shown to reduce tumour growth in several cancer preclinical models, suggesting that complement could be an attractive therapeutic target. Hypoxia (low oxygen) is frequently found in solid tumours and has a profound biological impact on cellular and non-cellular components of the tumour microenvironment. In this review, we focus on hypoxia since this is a prevailing feature of the tumour microenvironment that, like increased complement, is typically associated with poor prognosis. Furthermore, interesting links between hypoxia and complement have been recently proposed but never collectively reviewed. Here, we explore how hypoxia alters regulation of complement proteins in different cellular components of the tumour microenvironment, as well as the downstream biological consequences of this regulation.

INTRODUCTION

The tumour microenvironment is composed of a complex set of immune and non-immune components.[1,2] Together the components of the tumour microenvironment promote a pro-tumorigenic milieu by secreting pro-inflammatory molecules as well as growth factors and extracellular matrix degrading enzymes.[2–4] Complement is an innate immune component of the tumour microenvironment that has received increasing attention in recent years.[5,6] Complement has typically been regarded as a set of soluble and membrane-bound proteins involved in the first line of defence against pathogenic organisms.[7,8] Almost all immune cell types have been found to express complement proteins and importantly tumour and stromal cells may also produce several complement factors.[6] The exact details underlying the complex regulation of complement activation in the tumour microenvironment are not completely understood but elegant studies have highlighted that high levels of complement activation products as well as regulators in cancer cells often are associated with decreased prognostic outcome.[6,9]

"Non-cellular" factors in the tumour microenvironment can also have a negative impact on patient prognosis and are involved in regulating the biological behaviour of the different components of the tumour microenvironment, including complement.[10–14] A clear example of a prevalent "non-cellular" factor of the tumour microenvironment, pervasive amongst almost all solid tumours, is hypoxia. Tumour hypoxia refers to the low oxygen tensions that arise due to the imbalance between oxygen supply and demand in the aberrantly-perfused tumour.[15,16] Hypoxia is well known to influence several cellular and pro-tumourigenic components of the tumour microenvironment.[1] In

this review we describe how the impact of hypoxia on cellular members of the tumour microenvironment affects complement regulation and how complement dysregulation can contribute to tumour progression. A detailed description of cells associated with the tumour microenvironment is outside the scope of this review and has been described elegantly by Hanahan and Coussens.[2] Instead, focus will be placed on those components of the tumour microenvironment that are well known to be influenced by hypoxia.

The complement system in cancer

The complement system is a network of soluble serum proteins, membrane-bound receptors and regulatory proteins that interacts with both innate and adaptive immune system effectors. [8] Complement serves as an intermediary between the two branches of the immune system to eliminate pathogens or "altered-self" by opsonisation, inflammatory response mounting and direct cell lysis.[8] Opsonisation refers to the process of tagging of altered species leading to engulfment by phagocytes. There are three major pathways to complement activation: classical, lectin and alternative, each of which is initiated by different signalling stimuli but converges at the downstream cleavage of complement component 3 (C3) to produce C3a and C3b (Figure 1).[19] C3b production facilitates formation of the C5 convertase which cleaves C5 into C5a and C5b, the latter of which initiates assembly of the membrane attack complex (MAC) on the

Figure 1.Overview of the complement pathways. The classical pathway is initiated by recognition of immune complexes while the lectin pathway is initiated by recognition of carbohydrates or glycans on the pathogen's surface, resulting in the convergence of the two pathways with cleavage of C2 and C4 to generate C2a, C2b, C4a and C4b. C2a and C4a remain soluble to act as inflammatory mediators while C2b and C4b form the C3 convertase C4b2b. C3 is then cleaved by C4b2b to generate anaphylatoxin C3a and C3b. C3b can serve as an opsonin, or, upon binding with the C3 convertase, forms the C5 convertase C4b2b3b. C5 is then cleaved producing C5a and C5b. C5a is another potent inflammatory mediator while C5b initiates MAC formation and results in cytolytic activity. The alternative pathway can be initiated by (a) spontaneous hydrolysis of soluble C3 producing C3(H_2O), or (b) non-covalent binding of properdin to the target membrane. C3(H_2O) binds with Factor B and Factor D which cleaves Factor B into Ba and Bb. C3(H_2O) and Bb form one of the alternative pathway C3 convertases. Properdin binds with C3b and Factor B, cleaving Factor B to produce the other alternative pathway C3 convertase C3bBb. C3 cleavage is amplified in the alternative pathway and accounts for approximately 70% of complement activity. The C3 convertase C3bBb binds with another C3b fragment forming the alternative pathway C5 convertase C3bBb3b.[6–8,17,18] MAC, membrane attack complex.

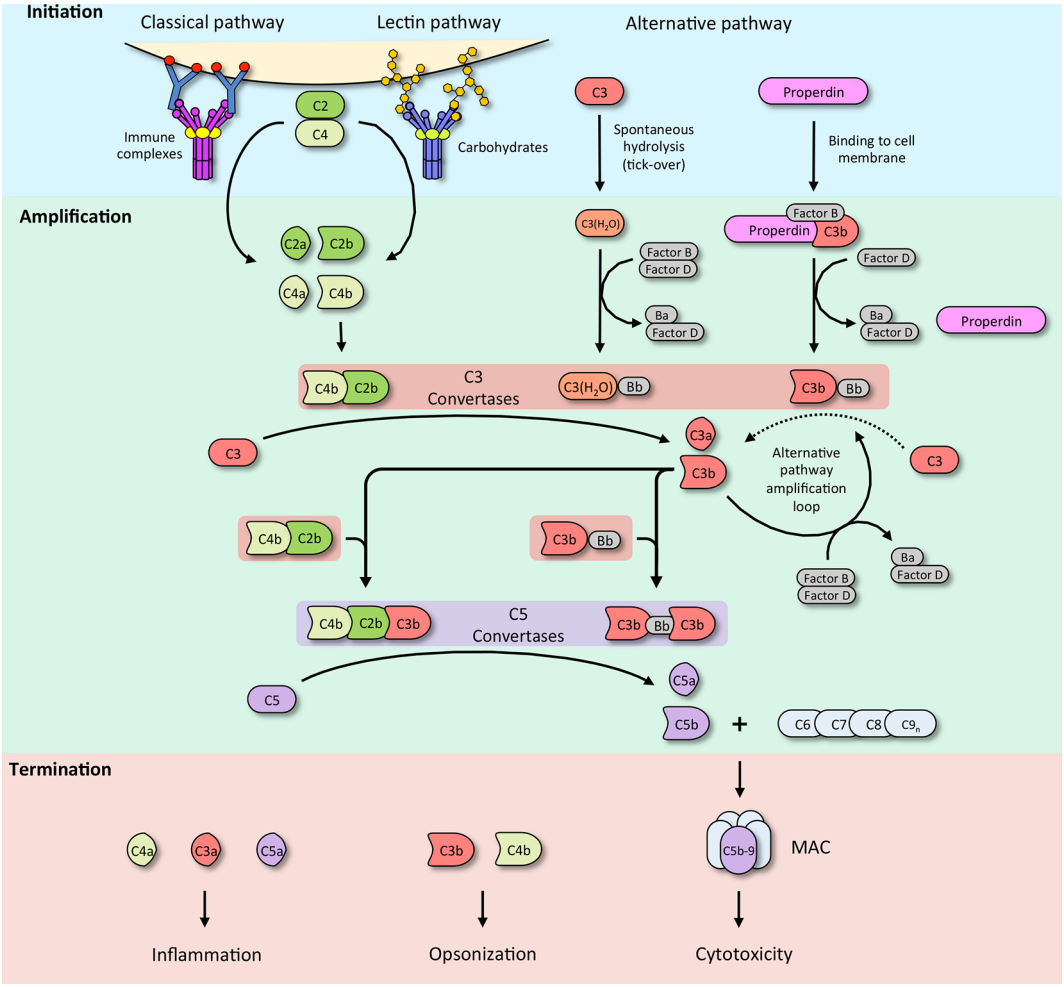
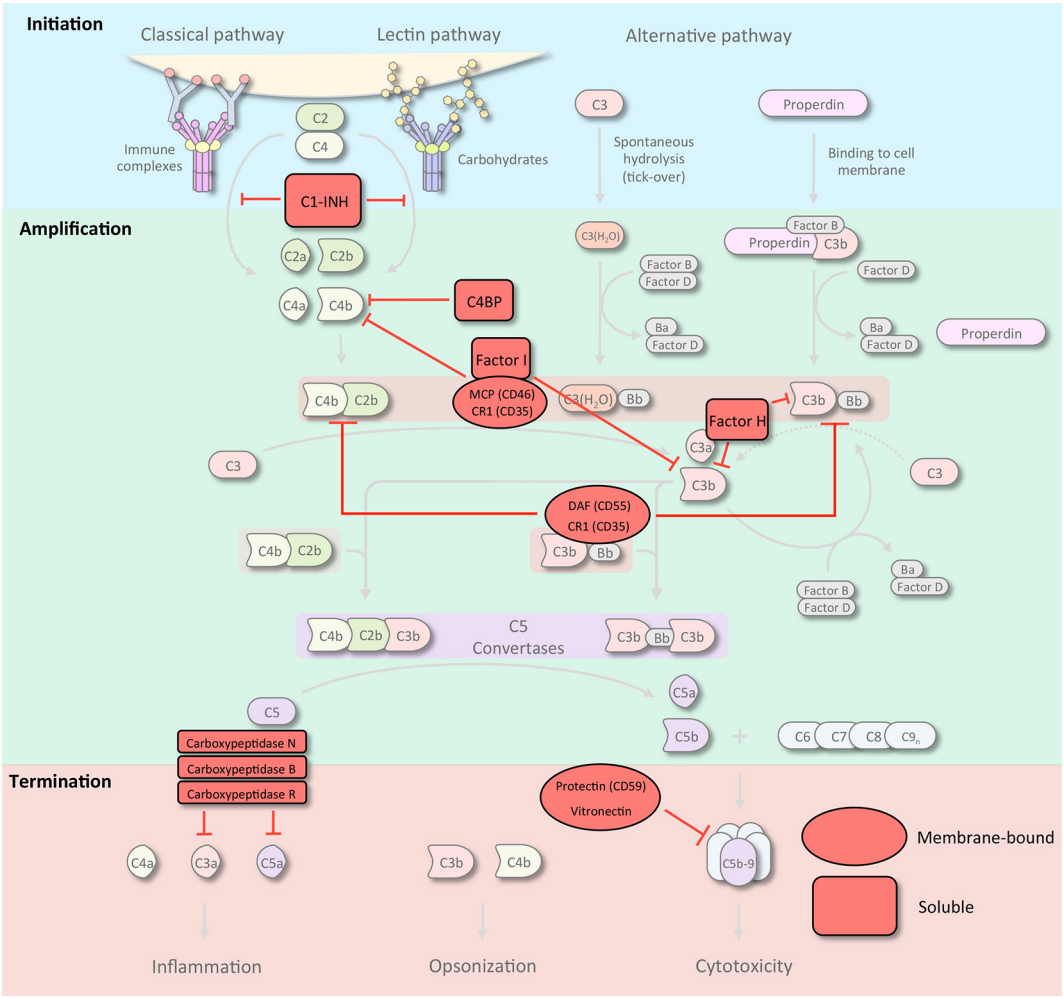


Figure 2.Schematic representation of complement regulators. Effects of the complement system are controlled by membrane-bound and soluble regulators. Complement regulators either inhibit proteases or accelerate the decay of certain complement components. C1 inhibitor (C1-INH) inhibits the serine proteases that cleave C4 and C2. C4 binding protein (C4BP) accelerates the decay of the classical pathway C3 convertase. Membrane cofactor protein (MCP) and CR1 are cofactors for Factor I, which degrades C3b and C4b fragments. Factor H degrades alternative pathway convertases. DAF and CR1 also accelerate decay of C3 convertases. Protectin and vitronectin prevent assembly of the MAC and the carboxypeptidases N, B and R degrade C3a and C5a to their less potent forms.[17,23,24] MAC, membrane attack complex.



pathogen's surface, thereby lysing the cell by perforating the cell membrane (Figure 1).[20] C5a is the most potent anaphylatoxin of the complement system, 20-fold more potent than C3a and 2500-fold more than C4a.[17] Anaphylaxis can induce inflammation and lead to secretion of IL-6 and TNF-α. Anaphylaxis can also result in promotion of phagocytosis, and movement of lymphocytes into neighbouring lymph nodes.[21,22] The effects of complement are tightly regulated by complement regulatory proteins which serve to accelerate degradation of complement components and convertases as well as inhibit MAC formation (Figure 2).[23]

Despite the defensive capabilities of the complement system, only limited evidence has demonstrated direct complement-mediated elimination of nascent tumours.[9] However, tumours have been found to exhibit complement-avoidance mechanisms, indirectly supporting a tumour-suppressive role for complement during cancer initiation.[24–26] The expression of complement regulators is upregulated in a variety of cancer types which suggests a selective pressure in favour of protection against complement recognition and complement-mediated attack.[27–29] Additionally, levels of complement activation fragments are upregulated in cancer patients suggesting that recognition of tumours by immune complexes triggers complement activation.[30]

Complement also has an immunomodulatory role in potentiating the responses of other immune cells involved in immunosurveillance and other tumour defence mechanisms.[31,32] Complement activation can enhance adaptive immune responses by enhancing dendritic cell uptake, antigen presentation and lowering the threshold for B cell activation.[33,34] Other studies have shown that the presence of complement receptors for C3a and C5a (C3aR and C5aR1) are involved in CD4+ T-cell survival and differentiation.[35,36] In this context, complement activity has been shown to contribute to cancer vaccine responses with promising results.[37]

However, the canonical understanding of complement acting solely as a protective system has been challenged by a growing body of evidence showing that complement activation may also promote tumourigenesis. Notably, in a murine model of colitis-associated colon cancer using azoxymethane and dextran sulphate sodium , loss of complement proteins C3, C3aR1 and C5aR1 was found to suppress tumourigenesis formation.[38] The authors proposed that C5a-dependent induction of the IL-1β/IL-17A signalling axis was responsible for this effect.[38] The link between inflammation and tumour progression is well recognised, and the fact that complement is upregulated in patients with cancer may allow nascent tumours to productively use anaphylatoxin-induced inflammation.[39] Furthermore, C3aR and C5aR1 signal through the PI3K-AKT pathway in an autocrine manner thereby facilitating tumour cell proliferation.[40] Sublytic doses of MAC deposition on the surface of cancer cells, facilitated by the upregulation of complement regulators, have also been proposed to promote cell proliferation and resistance to apoptosis.[18]

Complement system activation within the tumour microenvironment therefore has a multitude of roles. While the canonical defensive capabilities of complement-mediated attack were originally hypothesised to facilitate immune detection and clearance of tumours, a growing body of evidence suggests that increased expression of complement proteins is typically associated with poor prognosis and likely serves tumour promoting roles.[6] Significantly, the interest in targeting complement either alone or in combination with other therapies warrants a more exhaustive study of the complexities underlying complement system regulation in the tumour microenvironment.

Hypoxia in the tumour microenvironment

Hypoxia, an oxygen deficiency in tissues, is a common feature of the tumour microenvironment.[15,41] Hypoxia usually exists as a range of O_2 concentrations typically ranging from 2 to <0.1% in tumours as opposed to 9–5 to 1% in normal tissues.[42] Tumour hypoxia is often classified as either acute or chronic, both types typically originating as a consequence of the disorganised, inefficient and torturous tumour vasculature. Acute hypoxia, for instance, can occur due to temporary blood flow shutdown following obstruction of vessels. Chronic hypoxia can occur for several reasons, including cell proliferation beyond the oxygen diffusion distance from tumour microvessels.[43] The low oxygen state results in pro-survival gene expression changes that result in a plethora of effects including increased tumour angiogenesis, invasion and metastasis.[44–47] Consequently, hypoxia correlates with a negative patient prognosis.[10,48] Furthermore, hypoxia is associated with reduced effectiveness of several treatments including radiotherapy.[41,49,50] Many of these changes are driven by a family of transcription factors called the hypoxia-inducible factors (HIFs).[46,51] Though there are several different HIF isoforms, HIF-1 has been the primary target for study in gene expression alterations associated with cancer.[52–55] HIF-1 is a heterodimer that consists of a constitutively expressed HIF-1β subunit and a more tightly regulated HIF-1α subunit.[56,57] Under normoxic conditions, HIF-1α is targeted for degradation via oxygen-dependent degradation that involves hydroxylation and ubiquitination leading to proteolysis of the subunit.[58–63] In hypoxic conditions, HIF-1α is not ubiquitinated and is able to interact with the beta subunit, forming the heterodimer.[56] This occurs because of the oxygen sensitivity of prolyl hydroxylase domain containing proteins (PHDs) which hydroxylate two residues on HIF-1α, P402 and P564, which are necessary for von Hippel-Lindau disease tumour suppressor binding and ubiquitination.[58–60,64,65] PHDs have a relatively high Km value of 230–250 μM for oxygen, ensuring that given adequate levels of other substrates and cofactors, oxygen is the controlling factor in PHD activity.[66] A functional HIF complex binds to hypoxia-responsive elements and induces the expression of a number of genes that alter the cell's ability to adapt to the low oxygen environment.[46,67,68]

Besides cancer cells, the tumour microenvironment is composed of stromal cells, including cancer-associated fibroblasts, immune cells, endothelial cells and pericytes. Hypoxia affects the biological responses of all of these tumour microenvironment cells in a manner that usually potentiates tumour progression.[1,2] For example, hypoxia within the tumour microenvironment stimulates HIF-dependent angiogenesis through recruitment of endothelial cells and pericytes.[69] This process also enables recruitment of bone marrow derived cells. Growth factor signalling coupled with extracellular matrix remodelling by recruited stromal cells can further facilitate tumour progression.[1,3]

Hypoxic regulation of the complement system in tumour cells

Regulation of both complement component and regulator proteins has been described in tumour cells exposed to hypoxia (Figure 3). Early reports indicated that hypoxia-induced messenger RNA (mRNA) expression of central complement component C3 in liver cancer (HepG2) cells.[70]

Non-small cell lung cancer cells exposed to hypoxia (1% O_2) have more recently been described to express reduced levels of complement regulators CD46, *CD55* and CD59.[28] Decreased secretion of factor I and factor H was also reported in non-small cell lung cancer cells exposed to hypoxia.[28] The authors of this study hypothesised that altered levels of complement regulators under hypoxic conditions could lead to changes in complement-mediated lysis since increased C3b and C9 deposition coincided with altered expression of complement regulators. However, no significant changes in complement-mediated attack were reported.[28]

Interestingly, the use of antigens used/produced during immunodetection of tumour hypoxia when 2-nitroimidazoles (*e.g.* pimonidazole) bind to hypoxic cells has been proposed as a means of stimulating complement-mediated lysis of tumour cells.[71] This hypothesis was tested using rabbit complement as a means of lysing pimonidazole-labelled V79-4 cells in the presence of monoclonal antibody recognising reductively activated pimonidazole protein adducts. In this system the authors reported complement-mediated lysis of tumour cells at pimonidazole concentrations below those known to affect cell viability.[71] It would be interesting to test this concept *in vivo* to assess the

Figure 3.Table summarising main complement protein expression changes reported in cells exposed to hypoxia *in vitro*. mRNA and protein expression changes in the reported cell lines are shown. References are given in the last column. HUVEC, human umbilical vein endothelial cell; mRNA, messenger RNA.

Complement System Member	Response to Hypoxia (mRNA)	Response to Hypoxia (Protein)	Cell Line	Reference
C3	Increase		HepG2	70
CR1 (CD35)	Increase		HUVEC	97
MCP (CD46)	Decrease	Decrease	H2087, H358	27
		Increase	HUVEC	100
DAF (CD55)	Decrease	Decrease	H2087, H358	27
	Increase	Increase	T84, Caco-2	94
			HUVEC (protein only)	100
Protectin (CD59)	Decrease		H358	27
Factor H		Decrease	H2087, H358	27
Factor I		Decrease	H2087, H358	27

possibility of targeting "hypoxia-specific" antigens for induced complement-mediated lysis.

Interplay between complement and cellular components of the tumour microenvironment

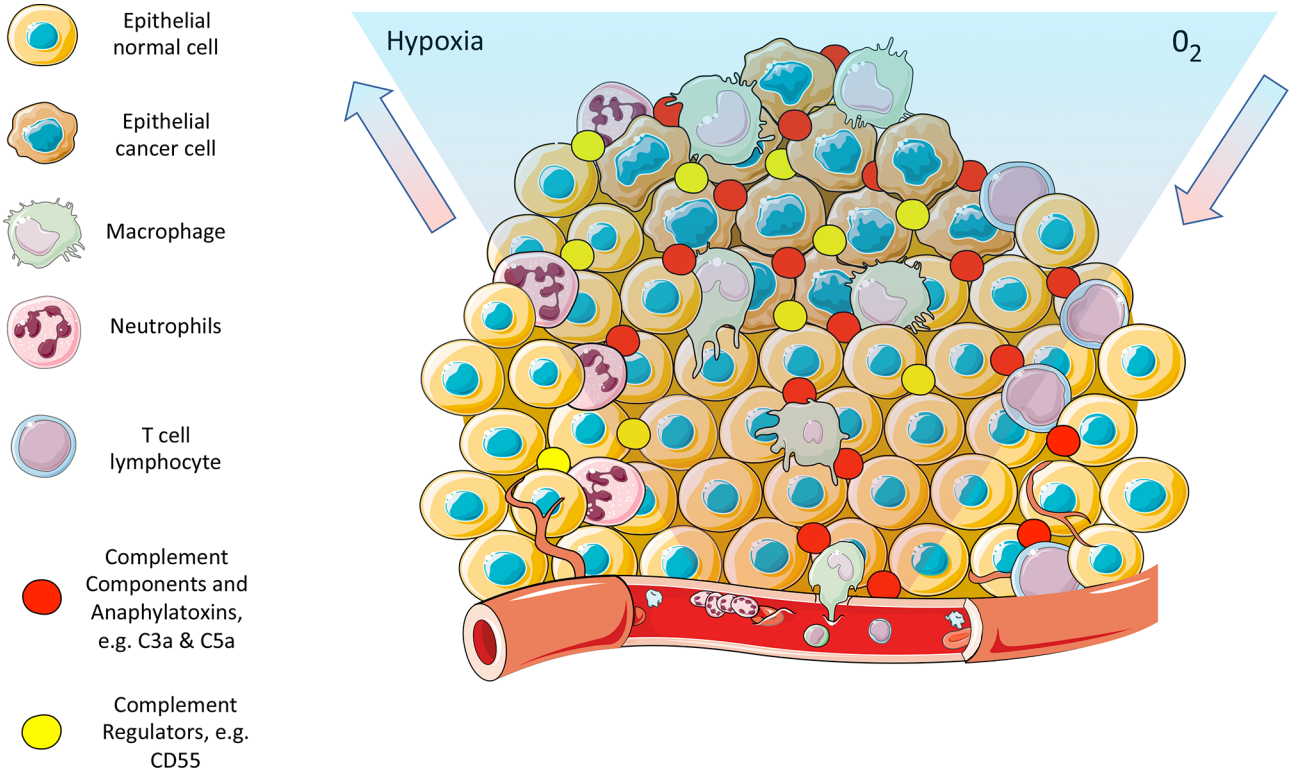
T-cells

Increasing interest in the study of hypoxic regulation of T-cell function has highlighted the complexity of the effects of hypoxia and hypoxic signalling on this cell population.[72,73] While low oxygen concentrations have been reported to increase CD8+ T-cell cytotoxicity, hypoxia can also decrease the production of effector and proliferative cytokines in T-cells.[74–76] Interestingly, a major impact of hypoxia on T-cell function stems from the metabolic changes associated with hypoxic environments resulting in an acidic pH. For instance, T-cell proliferation induced by IL-2 is arrested at pH 6.7. This is a problem in hypoxic tumours which can typically have an extracellular pH range of 5.8–6.5.[73,77] Overall, the effects of hypoxia on T-cell function and survival can therefore probably be considered to contribute to the development of an immunosuppressive microenvironment.[76,78] With respect to complement, a number of recent elegant studies have highlighted how complement activation in the tumour microenvironment further contributes to the immunosuppressive phenotype.[6,79] C3 was recently found to have an inhibitory effect on CD8+T-cells through IL-10 inhibition. Increased IL-10 expression in C3-deficient mice renders mice resistant to tumour development in a IL-10- and T-cell-dependent manner.[80] C5a further leads to a decrease in cytotoxic CD8+ T-cell responses through recruitment of myeloid-derived suppressor cells (MDSCs).[79] Increased T-cell suppressive capabilities are associated with C5a-mediated regulation of reactive oxygen and nitrogen species in MDSCs.[79] Hypoxia also regulates reactive nitrogen species through hypoxic induction of inducible nitric oxide synthase which increases reactive nitrogen species, especially peroxynitrite.[81] Increased reactive nitrogen species have a number of consequences, including nitration of CCL2 which diminishes effector lymphocyte recruitment function while retaining suppressive myeloid cell chemo-attractant capabilities.[82]

Macrophages

Macrophages recognise "molecular patterns" on the surfaces of pathogens in a process involving multiple ligand-receptor interactions.[83] Opsonins such as complement component cleavage products play an important role in this process by orchestrating pathogen internalisation during phagocytosis.[84,85] Links between complement activation and tumour promoting macrophage recruitment have been established in the context of PTX3 deficiency, which leads to complement activation and increased CCL2 production. Importantly these phenotypes are associated with increased susceptibility to certain mesenchymal and epithelial tumours.[39] The *PTX3* gene is silenced by methylation in certain cancers further increasing the clinical relevance of the association between complement regulation and macrophage recruitment.[39]

Figure 4.Interplay between hypoxia and complement in the tumour microenvironment. As tumour cells grow away from functional blood vessels, oxygen concentrations decrease and hypoxia develops. Hypoxia creates an immunosuppressive environment including decreased functional CD8+ T-cells and M2 polarised macrophages. Hypoxia also alters the expression of complement proteins and regulators on both tumour and endothelial cells in the tumour microenvironment. Dysregulation of complement proteins contributes to immunosuppression and can promote tumourigenesis.[1,28,39,45,70,74–76,78,79,87–89] Figure adapted from.[90]



C3a and C5a receptors are expressed on the cell surface of macrophages and the binding of ligands for these receptors has also been suggested to modulate angiogenesis.[7,86] The association between complement components, macrophages and angiogenesis suggests crosstalk between cell types and processes in the tumour microenvironment intimately linked to hypoxic signalling (Figure 4). Hypoxia and HIF-signalling are indeed critical for macrophage polarisation and deletion of either HIF-1α or 2α in macrophages reduces tumour growth.[74,87]

Neutrophils

Recruitment of immune cells such as neutrophils and monocytes can result in induction of hypoxia at sites of inflammation.[91] Hypoxia has indeed been associated with inflammatory conditions, some of which have been proposed to predispose to certain cancers. This is the case for colitis and inflammatory bowel disease.[92–94] In these inflammatory conditions, transepithelial migration of neutrophils is a marker of mucosal inflammation.[95] Several proteins are implicated in the interaction between neutrophils and epithelial cells including complement regulator *CD55*. *CD55* functions in the later stages of transepithelial migration by facilitating the release of neutrophils from the epithelial surface.[96] *CD55* is expressed on the apical membrane of mucosal epithelial cells. Importantly, HIF-binding sites are found in *CD55* and *CD55* expression was found to be hypoxia inducible (Figures 3 and 4).[97] Therefore, hypoxia, through *CD55* induction, may enhance neutrophil transepithelial migration and promote neutrophil clearance from the epithelial surface in conditions predisposing to cancer.[97]

A further role for complement in neutrophil function has been described in melanoma where C3a/C3aR1 signalling has been implicated in tumour progression by inhibiting CD4+ T-cell and neutrophil responses.[88] C3aR1 was also found to be upregulated in intestinal neutrophils in a murine model of intestinal tumourigenesis (using $APC^{min/+}$ mice), where C3aR1 signalling promoted tumourigenesis through triggering neutrophil extracellular traps.[98]

Endothelial cells

Endothelial cells are critical for angiogenesis, the process of new capillary growth from established blood vessels. Angiogenesis is important for nutrient and oxygen supply and is a process induced following periods of hypoxia.[44,45] Whether or not complement activation promotes or inhibits angiogenesis is controversial and seems to depend on the model and disease being studied.[9] Some of the controversy may stem from the dual role described for some complement proteins expressed on endothelial cells. CR1 for example has been found to be expressed in primary human umbilical vein endothelial cells (HUVECs) and hypoxia (1% O_2) induces CR1 protein expression.[89] CR1 is both a receptor for C1q and a regulator of the complement system suggesting that hypoxic induction of CR1 could have positive or negative effects

on complement activation on these cells.[89] Interestingly, CR1 in HUVECs was found to be present intracellularly and could act as a cofactor for factor I-mediated cleavage of iC3b to C3c and C3dg. soluble CR1 on the other hand was found to inhibit binding of C3b and immune complexes to hypoxic HUVECs and it was suggested that a portion of CR1 is expressed on the extracellular membrane.[89] A recent study reported C1q expression in the stroma and vascular endothelium of tumours correlating with increased vascular density and lung metastasis. Importantly, B16 melanoma tumours display decreased tumour growth in C1q-deficientmice and these effects were not attributed to differences in immune cell infiltration.[99]

Classical complement activation has also been proposed to occur on endothelial cells following hypoxia/reoxygenation *in vitro*.[100–102] Most of these studies have used HUVECs exposed to hypoxia (1% O_2) or hypoxia followed by reoxygenation at 21% O_2 as a model of hypoxia/reoxygenation of endothelium exposed to ischaemia reperfusion injury.[100–102] During initial studies, complement activation was found to occur in the presence of serum-activated complement.[101] Interestingly, reoxygenation-induced complement activation was subsequently shown to be inhibited by membrane permeable free radical scavengers.[102] Intriguingly, surface expression of complement regulators *CD55* and CD46 was also found to be increased in early studies[101] (Figure 3). Furthermore, a subsequent study reported that C3d deposition in this model was thought to occur on reoxygenated apoptotic cells and this appeared to occur in the absence of antibodies or serum factors.[100] In support of these findings C3 activation was abolished after treatment with caspase inhibitor treatment.[100] It would be interesting to investigate if complement is also activated on other cells in the tumour microenvironment following the induction of apoptosis (either during reoxygenation or following treatment with apoptosis inducing agents such as chemo- or radiotherapy).

THE COMPLEMENT SYSTEM IN THE CONTEXT OF CANCER THERAPY

The success of various cancer therapies has been linked, in part, to the effects of complement activation. The efficacy of monoclonal antibody (mAb)-based cancer therapy, for instance, is due in part to the ability of the antibody to induce complement-dependent cytotoxicity which results in tumour cell killing.[103,104] The potency of mAbs in therapeutic regimens stems from the dual ability of antibodies to decrease tumour proliferation by blocking oncogenic signalling and to promote cytotoxicity.[104–106] Antibody binding to tumour antigen can result in activation of the complement cascade via the classical pathway which results in MAC assembly, antibody-dependent cell-mediated cytotoxicity and complement-dependent phagocytosis.[103] Anaphylatoxic inflammatory mediators released as a result of complement activation enhance the response by facilitating recruitment of phagocytic cells.[107]

Importantly, targeting complement has recently been proposed as means of improving tumour immune responses.[73,80,108] Treatment with a C5aR antagonist alone reduced tumour growth to levels comparable to those achieved following treatment with chemotherapy agent paclitaxel.[79] With the increasing interest in immune checkpoint inhibitors, the potential for targeting complement, particularly at the level of C5a/C5aR axis, together with current immunotherapy approaches, such as programmed death 1/programmed death ligand 1 (PD-1/PD-L1) antibodies, has been explored.[80,108] Interestingly, increased complement activation, including, C5a was found to be produced in tumours following treatment with anti-PD-1 antibodies.[80] Remarkably, however, increased anti-tumour immunity following complement inhibition (such as with C5aR1 antagonists) was found to be independent of the PD-1/PD-L1 immune checkpoint pathway. These findings have led to the suggestion that complement receptors such as C5aR1 and C3aR1 could be a new class of immune checkpoints to be targeted.[80]

Furthermore, it has been found that radiotherapy elicits C3a and C5a upregulation within the tumour microenvironment, potentially aiding in the anti-tumour response.[109] However, seemingly contradictory results have been published as it was shown that complement inhibition enhances anti-tumour response after fractionated radiation therapy.[110]

CONCLUSION

Complement activation and hypoxia have both been shown to facilitate tumour progression by altering the function of tumour microenvironment components.[4,6,99,111–113] Complement-effector functions alter cellular components known to be modulated by hypoxia such as tumour cells, endothelial cells, T-cells, macrophages and neutrophils.[1,6] Interestingly, complement imbalances in these cells have also been associated with hypoxia-associated processes such as increased migration, angiogenesis and immunomodulation.[1,6,72] Hypoxia has been directly shown to alter regulation of complement proteins not only in cancer cells but also in endothelial cells.[28,89,97,100] It is tempting to speculate that hypoxia-mediated regulation of complement in other cellular components such as T-cells, macrophages and MDSCs might be described in the future given the already established links between these cell types and complement. T-cells, macrophages and MDSCs have emerged as critical immune components of the tumour microenvironment so any potential interplay between hypoxia and complement in these cells could have important biological consequences for tumour progression.[39,79] Importantly, targeting both hypoxia (and hypoxia-associated processes) as well as complement has been proposed as a means of improving tumour responses both from an immune and non-immune standpoint.[80,108,114] It would be interesting to explore whether targeting both complement and hypoxia might yield improved clinical responses.

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